PLANT REGENERATION FROM SWEETGUM *(LIQUIDAMBAR STYRACIFLUA)* NODULE CULTURES AND GENETIC TRANSFORMATION BY MICROPROJECTILE BOMBARDMENT

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Abstract: Sweetguin *(Liquidambar styraciflua)* nodule cultures were established from seedling hypocotyls and proliferated in liquid Blaydes' medium containing 0.1 mg/I TDZ and 0.01 mg/I 2,4-D. Shoots differentiated from the nodules in liquid Blaydes' medium containing BA (I mg/I), BA (0.5 mg/I) and NAA (0.01 mg/I), or BA (0.5 mg/I), NAA (0.01 mg/I), and TDZ (0.05 mg/I) in the light. Differentiating shoots required a period of dark treatment for further development on solid medium containing 1 mg/I BA. Elongated shoots were harvested and the cut ends were soaked in 10 mg/I IBA solution prior to planting in potting mix for ex vitro rooting. Roots differentiated and leaves expanded in two weeks. Sweetgum nodules were stably transformed by microprojectile bombardment using a 7.4 kb plasmid, pTRA 140, harboring CaMV 35S-HPH and CaMV 35S-GUS. Evidence that nodules growing in the presence of hygromycin B were stably transformed was provided by PCR analysis and ß-glucuronidase activity. Sweetgum shoots differentiated in liquid medium in the presence of hygromycin B. Shoots transferred to solid medium lacking hygromycin-B elongated and displayed ß-glucuronidase activity in their expanding leaves and stems. PCR analysis confirmed the presence of GUS gene in shoots. Transgenic shoots initiated roots and showed leaf expansion two weeks following planting in potting mix.

Key words: Genetic transformation, nodule culture, microprojectile bombardment, sweetgum, *Liquidambar styraciflua.*

INTRODUCTION

Liquidambar styraciflua L. (sweetgum) is a major hardwood species of the southern United States which is gaining in commercial importance. Incorporation of foreign DNA into sweetgum cells and the subsequent regeneration of transformed plants hold promise for overcoming major obstacles in the conventional genetic improvement of woody perennials, e.g., the transfer of specific genes from other taxa, or modified expression of specific native genes.

Recently, leaf tissue derived from aseptic shoot tip cultures (Sullivan and Lagrimini,1993) and young leaf explants (Chen and Stomp,1992) were transformed through *Agrobacterium-mediated* gene transfer, and transgenic sweetgum trees were regenerated via adventitious shoot induction. *Agrobacterium-mediated* transformation has been pursued in this species due to the immediate initiation of cell division upon wounding and the susceptibility of the species to the bacterium (Stomp, 1991). However, complex host-parasite interactions (Sellmer and McCown, 1989), unanticipated variation (even within a species and clone) in sensitivity to infection (Potrykus, 1990; Binns, 1990; Bergmann and Stomp, 1992, 1994), and low suitability for use with established embryogenic cultures (Merkle et al., 1997) or nodule suspension culture systems of sweetgum could limit the use of the *Agrobacterium-mediated* gene transfer.

On the other hand, biolistic transformation, which involves the acceleration of small metal particles coated with DNA to deliver DNA fragments into plant cells, has been shown to be applicable to a wide range of plant species including forest tree species such as hybrid *Populus* (McCown et al., 1991), yellow-poplar *(Liriodendron tulipifera)* (Wilde et al., 1992; Merkle and Sommer, 1986), and white spruce *(Picea glauca*)(Ellis et al., 1993). The biolistic method has been well integrated

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with embryogenic cultures (Wilde et at, 1992; Ellis et at, 1993) and nodule cultures (McCown et at, 1991), giving the potential to produce transgenic trees on a large scale (Bonga and Von Aderkas, 1992).

The goal of the current study was to establish nodule cultures and to integrate genetic transforination by particle bombardment into this regeneration system for sweetgum, as a potential alternative to the embryogenic culture system. Genetic transformation by particle-bombardment has not yet been demonstrated with sweetgum.

MATERIALS AND METHODS

Nodule culture initiation and maintenance. Sweetgum *(Liquidambar styraciflua)* seeds from a bulked seedlot were surface-sterilized and germinated in vitro on a modified Risser and White's (1964) medium (Sommer and Brown, 1980). Hypocotyl segments were collected when the seedlings were 2 weeks old and cultured on a modified Blaydes' (Witham et al. 1971) medium supplemented with 1 mg/I 2,4-D. Cultures were maintained under a 16-h photoperiod (100 $\mu mol/m^2/s$) provided by cool-white fluorescent lamps at 25 ± 2°C.

Green nodules proliferating along with callus from the hypocotyl cultures were collected and cultured in liquid Blaydes' medium containing the following combinations of TDZ and 2,4-D: TDZ alone at 0.001, 0.01, 0.1, 0.5, or 1 mg/I, 2,4-D alone at 0.01, 0.1, I, or 10 mg/I, or factorial combinations of TDZ at 0.01, 0.1, or 1 mg/I with 2,4-D at 0.001, 0.01, 0.1, or 1 mg/l. After 6 weeks, nodule cultures were evaluated for response to the different plant growth regulator (PGR) treatments. Based on these results, for subsequent culture, green nodules were collected and proliferated in liquid Blaydes' medium containing 0.I mg/I TDZ and 0.1 or 0.01 mg/I 2,4-D. At 3 week intervals, small (less than 3 mm in diameter), green, nodular aggregates were collected and subcultured at an inoculum density of 1 ml of settled nodule volume (SNV) per 40 ml of liquid medium in 125 ml Erlenmeyer flasks. Cultures were maintained on an orbital shaker at 100 rpm under the same conditions used for nodule initiation.

Shoot regeneration and plantlet production. Nodules were induced to produce adventitious shoots in liquid medium containing one of the following combinations of growth regulators: TDZ (0.2 mg/I) ; BA (0.5 mg/I) and NAA (0.0I mg/I); TDZ (0.5 mg/I), BA (0.5 mg/I) and NAA (0.01 mg/I). In a preliminary experiment, shoots proliferated on solid medium in the light first turned red and then darkened and died when cultures were maintained in the light. When maintained in the dark, most shoots remained alive. Therefore, nodules showing shoot differentiation in liquid medium were transferred to solid mediuin containing 1 mg/I BA and subjected to 0, 2, 4, or 6 weeks of dark treatment. Cultures were transferred to fresh medium every 6 weeks. Shoots longer than 1 mm were counted at I0 weeks of culture. Data were analyzed by GLM (SAS/STAT User's Guide, 1988). Elongated shoots greater than 2 cm were harvested and the cut ends were soaked in 10 mg/I IBA solution for 10 minutes prior to planting in potting mix for ex vitro rooting (Kim et al., I997).

Transformation and analysis of transgenic nodule cultures. The plasmid pTRA 140, harboring CaMV 35S-HPH and CaMV 35S-GUS (provided by Dr. Murai, department of plant pathology and crop physiology, Louisiana State University) was used for microprojectile bombardment. Plasmid DNA was loaded onto gold particles and bombarded onto nodule culture cells following the DuPont DNA preparation protocol (Fraley et al., 1984). Newly proliferating nodules were fractionated through 20 mesh (1.2 mm) and 40 mesh (0.38 mm) screens 2 weeks following subculture and maintained in fresh liquid medium supplemented with 0.1 mg/I TDZ and 0.01 mg/I 2,4-D or 0.1 mg/I 2,4-D for 3 days prior to bombardment. In preparation for bombardment, harvested nodules were placed on filter paper supports, blotted thoroughly with sterile blotting

paper and transferred to empty Petri dishes. One ml of SNV was used per plate. Following bombardment, nodules were transferred to non-selective semisolid medium with 0.1 mg/I TDZ and 0.01 mg/I 2,4-D, and incubated in the dark. After 2 days, nodules subsampled for histochemical GUS assay were stained with 5-bromo-3-chloro-3-indolyl-B-D-glucuronic acid solution (Jefferson et al., 1987). Cells were incubated at 25°C overnight and the number of blue spots was counted using a dissecting microscope. The remaining nodules were maintained for one week on non-selective medium and then transferred to selection medium containing the same PGRs, but with 5 mg/I hygromycin B. The concentration of hygromycin B was raised to 10 mg,/I at the third week of selection and to 15 mg/I at the eighth week of selection.

To provide evidence of stable transformation, nodules that had been under selection for 3 months were subjected to polmerase chain reaction (PCR) or histochemical GUS assay (Jefferson et al., 1987). For PCR, DNA was extracted from about 25 mg of fresh nodule cells by following the procedure of Stewart et al. (1993). To detect the relevant portion of the introduced DNA, a I200 bp sequence was amplified from a non-intron GUS gene by using the primers designed by Jefferson et al. (1986). Nodules (and later, shoot primordia, stems and expanding leaves) were assayed for GUS activity using the same histochemical assay used for transient expression. From the GUS positive transgenic shoots, DNA was extracted and used for PCR analysis.

Those nodule lines displaying evidence of stable transformation by GUS activity or PCR analysis were induced to produce adventitious shoots in the same liquid medium used for production of shoots from non-transgenic cultures, but containing 5 mg/I hygromycin B. When shoot clumps were transferred onto solid medium for elongation, hygromycin B was omitted.Elongated shoots were planted in potting mix for ex vitro rooting following the same procedure used for non-transgenic shoots.

RESULTS AND DISCUSSION

Nodule culture initiation and maintenance, Nodules, collected from callus masses from one to two months following hypocotyl culture initiation, were dense cell masses that were independent and formed spherical, cohesive units, fitting the description of nodules given by McCown et al. (1988). Suspension cultures initiated from these nodules were evaluated for the impact of the different PGR combinations on morphology, proliferation, and homogeneity after 6 weeks. Optimal proliferation and homogeneity of nodules were obtained with 0.1 mg/I TDZ and 0.01 or 0.1 mg/I 2,4-D. At levels of 2,4-D above 1 mg/I, with or without TDZ, cultures did not proliferate as green nodules. With TDZ alone most nodules turned green within a few weeks and some showed shoot differentiation. At TDZ concentrations above 1 mg/I, some nodule cultures turned dark and ceased further proliferation.

With levels of TDZ above 0.5 mg/I and 2,4-D above 1 mg/I, nodule size increased, resulting in highly heterogeneous suspension cultures. Treatment with 0.1 mg/I TDZ and 0.1 mg/I or 0.0I mg/I 2,4-D gave rise to three types of cell masses: (1) Green nodules, (2) white nodules, and (3) unorganized cell masses. With 0.1 mg/I TDZ and 0.01 mg/I 2,4-D, most of the nodules remained green and compact 3 weeks following subsequent 3-week subcultures (Type I). On the other hand, cultures maintained with 0.1 mg/I TDZ and 0.1 mg/I 2,4-D gave rise to all three types of cell masses mentioned above, but with only a small amount of green nodular cell masses at the end of 3 weeks of culture (Type II). Both types produced white or yellow daughter nodules within a week following subculture, but most of these turned green at the end of 3 weeks in Type I nodule cultures. Type II cultures lost their potential for adventitious shoot production within 6 months, but Type I cultures have maintained their potential for adventitious shoot differentiation for over I year. In addition, competent nodule cultures could be renewed by transferring the shoot differentiating nodules from BA-containing liquid medium (see below) to nodule proliferation medium containing 0.1 mg/I TDZ and 0.01 mg/I 2,4-D.

Shoot regeneration and plantlet production. In a preliminary study, shoot initiation from nodule cultures was initially observed following 6 weeks of culture in liquid medium containing one of the following combinations of growth regulators: TDZ (0.2 mg/I); BA (1 mg/I); BA (0.5 mg/I) and NAA (0.01 mg/1); TDZ (0.5 mg/I), BA (0.5 mg/I) and NAA (0.01 mg/I). However, shoot initiation was not observed on solid medium. In the current study, however, when nodules showing shoot differentiation were maintained in liquid mediuin, most of shoot primordia turned red and eventually lost their morphology with severe vitrification as described by Debergh et al. (1992). Therefore, they were transferred to solid medium to avoid further vitrification. Even on solid medium, most shoot primordia turned red with subsequent darkening and died in the light. When they were maintained in the dark, most shoot primordia retained their morphology, and new shoots emerged from the mass. This latter observation prompted us to test the effects of dark treatment on shoot formation and elongation on solid medium. A clear difference was shown in shoot formation between the control (no dark treatment) and the dark treatments (Fig. 2). Four weeks of dark treatment gave the highest shoot formation of 11 shoots per nodule cluster. When the differentiated shoots were exposed to light following dark treatment, they developed normally and leaves greened and expanded on solid medium containing 1mg/I BA. Dark treatment was apparently necessary to allow the vitrified shoots to recover physiologically for continued development on solid medium. All shoots transferred to potting mix rooted and leaves expanded two weeks following planting in potting mix(data not shown).

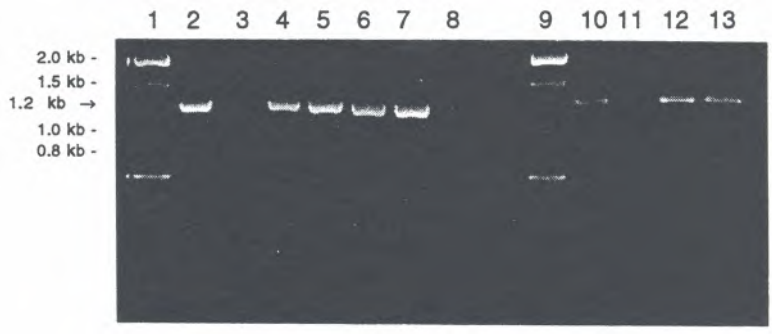

Figure 1. PCR analysis of the transgenic nodule cultures and the transgenic shoots for the integration of GUS gene. *Lanes 5-8* contained genomic DNA from GUS (+) independent Type II nodule cultures; *Lane 8* from GUS (-) Type II nodule cultures; *Lane 12* from GUS (+) Type I nodule cultures; *Lane 13* from GUS (+) shoots; *lanes 1* and *9* contained 100-bp DNA ladder; *lanes 3* and 11 represent the negative control of non-transformed sweetgum nodule cultures; *lanes* 2 and 10 contained the pTRA 140 plasmid of positive control containing GUS gene.

Transformation and analysis of transgenic nodule cultures. The highest levels of transient GUS expression observed were approximately 2,000 and 2,400 blue spots per 1 ml of settled nodule volume (SNV) from Type I and Type II nodules, respectively. Zero to three hygromycin-resistant nodules were recovered per plate, for a total of thirteen lines of hygromycin resistant nodules. Nine of these lines were Type II nodules. Of the five lines tested for the insert using PCR, four showed 1200 bp bands corresponding to the 1200 bp band from the positive control pTRA 140 plasmid (Fig. 1). Among the nine hygromycin-resistant lines, one showed only root differentiation in the presence of hygromycin in the medium containing 0.1 mg/I TDZ and 0.01 mg/I 2,4-D, and was GUS. On the other hand, the four hygromycin resistant lines derived from Type I nodules were all GUS positive. Of the four lines, one showed the typical morphology of Type I nodules and has maintained shoot differentiation in the liquid selection medium used for shoot induction. The other three lines resembled Type II nodules and did not produce shoot. As was the case for

non-transformed nodules, four weeks of dark treatment was optimal for shoot production, with an average of 14 shoots produced per nodule cluster (Fig. 2). At the time of rooting, most of the transgenic shoots, grown without hygromycin, were GUS positive in the stems and expanding leaves. However, some of the shoots were GUS negative even though they were derived from GUS positive, hygromycin resistant nodules. GUS positive transgenic shoots began to root *ex vitro* and leaves expanded two weeks following planting in potting mix. Five out of fifteen GUS positive transgenic shoots showed root initiation and leaf expansion within four weeks.

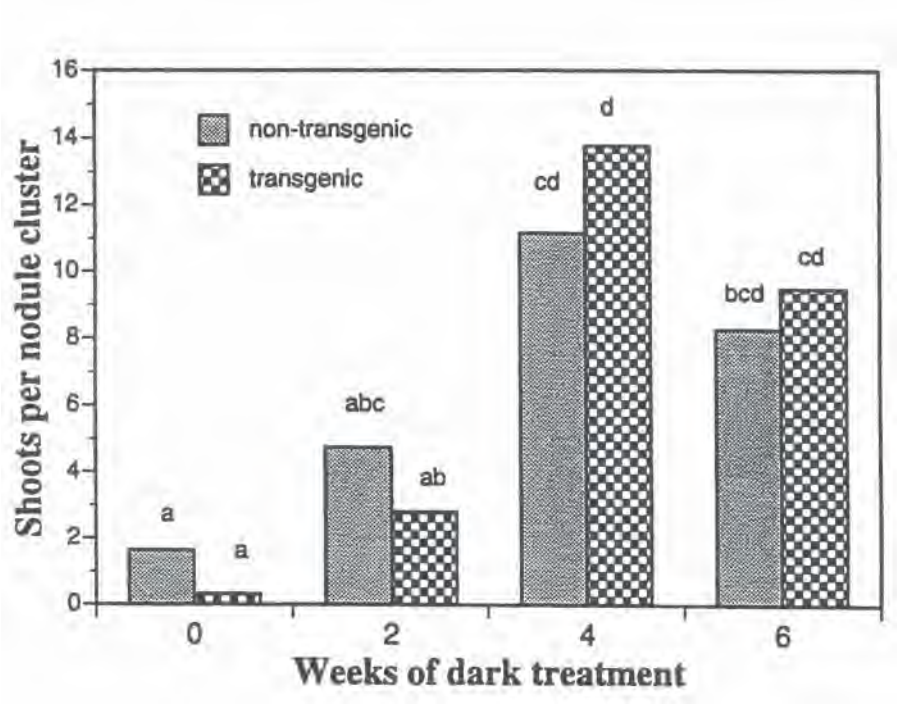

Figure 2. Effect of dark treatment on shoot production on solid medium from non-transgenic and transgenic nodules: Shoots greater than 1 mm were counted from 10 to 87 replicates at 10 weeks of culture. Means with the same letters are not significantly different at the 0.01 level using Duncan's multiple range test.

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